Electrostatic and Hydrophobic Interactions during Complex Formation and Electron Transfer in the Ferredoxin/Ferredoxin:NADP$^+$ Reductase System from *Anabaena*

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Received October 31, 1995[⊗]

Abstract: Transient kinetics and protein−protein binding measurements over a wide range of ionic strength (I) have been used to characterize the interactions occurring during complex formation and electron transfer (et) between recombinant ferredoxin (Fd) and both native and recombinant ferredoxin:NADP$^+$ reductase (FNR) from the cyanobacterium *Anabaena*. Between $I = 12$ mM and $I = 100$ mM, the dissociation constant (K_d) for the complex formed between oxidized Fd and oxidized native FNR increases from 4.5 to 8.1 μM, whereas K_d for the Fd complex with recombinant FNR increases from 0.3 to 3.3 μM. For both pairs of proteins, the ionic strength dependence of k_{obs} for the et reaction is biphasic. With native FNR, k_{obs} increases only slightly between $I = 12$ mM and $I = 100$ mM, whereas for recombinant FNR k_{obs} increases by about 4-fold over this ionic strength range. For both proteins, k_{obs} decreases monotonically above $I = 100$ mM. The dependence of k_{obs} on FNR concentration is linear for both pairs of proteins at $I = 12$ mM, with the second-order rate constant for recombinant FNR being about 3 times smaller than that for the native protein. In contrast, at $I = 100$ mM the k_{obs} values are the same for both protein pairs, and show saturation behavior with respect to the FNR concentration, indicating that et becomes rate-limiting at high FNR concentrations. Electrostatic analysis of the kinetic data above $I = 100$ mM allows a prediction of the ionic strength dependence of the K_d values, if electrostatic interactions are the only determinant of complex stability. The predicted dependence is dramatically larger than the observed one, indicating that hydrophobic interactions make an important contribution to complex stability. The differences in binding between native and recombinant FNR are ascribed to proteolytic cleavage at the N-terminus, which occurs during preparation of the native enzyme and which removes two positively charged residues, thereby decreasing the electrostatic interactions with Fd. The kinetic results are explained by assuming that formation of the oxidized protein−protein complex blocks the et site, and thus reaction only occurs between reduced Fd and free FNR. However, even after correction for the presence of the preexisting complex, the reactivity of FNR at $I = 12$ mM is significantly lower than that at $I = 100$ mM. This is ascribed to electrostatic effects which force the complex with reduced Fd to be less optimal, implying that hydrophobic interactions favor a more productive interaction between the two redox proteins.

Introduction

In green plant, algal, and cyanobacterial photosynthesis, ferredoxin (Fd) is the terminal electron acceptor from photosystem I. After being reduced, Fd, which is a small, acidic protein containing a single [2Fe−2S] center, interacts (in two one-electron steps) with ferredoxin:NADP$^+$ reductase (FNR), an FAD-containing flavoprotein which catalyzes the reduction of NADP$^+$ to NADPH according to eq 1, where the subscripts

$$2Fd_{red} + NADP^+ + H^+ \rightarrow 2Fd_{ox} + NADPH \qquad (1)$$

red and ox signify the reduced and oxidized states of the Fd, respectively. X-ray crystal structures for both Fd[1,2] and FNR[3] from *Anabaena* (derived respectively from the closely related strains 7120 and 7119) have been determined to high resolution, and both proteins have been cloned and overexpressed in *Escherichia coli*,[4,5] making them available in high quantities and making it possible to readily construct site-directed mutants.

In previous work from this laboratory,[6−8] we have investigated the electron transfer (et) mechanisms for the reactions of wild-type and site-directed mutants of recombinant Fd from the cyanobacterium *Anabaena* 7120 with native FNR (natFNR) isolated from *Anabaena* 7119. It was shown that the observed rate constant (k_{obs}) for the reduction of natFNR by Fd_{red} is linearly dependent on the concentration of FNR when the experiment is performed at low ionic strength (12 mM), but at higher salt concentrations k_{obs} showed a nonlinear dependence on FNR concentration. More recently, following the cloning and overexpression in *E. coli* of the *Anabaena* 7119 FNR gene,[5]

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[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

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S0002-7863(95)03662-6 CCC: $12.00

we have reported preliminary results on the et reaction between wild-type Fd and wild-type recombinant FNR (recFNR).[9] Similar to the previous results, at low ionic strength (I = 12 mM), k_{obs} for the reduction of both native and recombinant FNR_{ox} by Fd_{red} is linearly dependent on FNR concentration, whereas for the reaction at I = 100 mM a nonlinear FNR concentration dependence is observed. Generally, for a (minimal) two-step mechanism[10] such as that given in eq 2, curvature

$$Fd_{red} + FNR_{ox} \overset{K_d}{\rightleftharpoons} [Fd_{red}\cdots FNR_{ox}] \xrightarrow{k_{et}} Fd_{ox} + FNR_{red} \quad (2)$$

indicative of an approach to saturation is often observed, caused by a change in the rate-limiting step from complex formation to electron transfer. Indeed, such nonlinear behavior was obtained over a wide range of ionic strengths in the et reaction between natFNR and natFd from *Anabaena* 7119.[10] We have also reported measurements of the ionic strength dependence of k_{obs} for the reactions between Fd and natFNR and between Fd and recFNR, which showed a decrease in reaction rate constant occurring at low ionic strengths (I < 100 mM); this was interpreted in terms of the formation of nonoptimal complexes under these conditions.[6,9]

In order to further clarify the physicochemical mechanisms involved in these ionic strength effects, we have carried out a more detailed investigation of both the et kinetics and the protein−protein binding constants (K_d) for the reactions between wild-type Fd and both natFNR and wild-type recFNR over the ionic strength range I = 12−100 mM. The results obtained in this low ionic strength region are compared with a predicted ionic strength dependence for K_d based on an electrostatic model for the protein−protein interaction, derived from an analysis of the ionic strength dependence of the k_{obs} values at I > 100 mM using the Watkins equation.[11] We interpret this to indicate that both ionic and hydrophobic interactions occur during the protein−protein interaction, with hydrophobic forces becoming more prominent at higher values of I. At $I \geq$ 100 mM, the [Fd_{red}−FNR_{ox}] complexes involving both natFNR and recFNR become similar in reactivity, whereas at I = 12 mM both FNRs are less reactive. We ascribe these effects to a larger electrostatic interaction at low I which results in a less productive et complex, whereas the hydrophobic interactions between the two proteins favor a more productive et complex.

Methods and Materials

Wild-type Fd from *Anabaena* 7119 was overexpressed in *E. coli* and was prepared as described previously.[6] NatFNR from *Anabaena* 7120 was also isolated and purified by previously described methods.[12] The recFNR gene from *Anabaena* 7120 has been cloned and overexpressed[5] in *E. coli* PC 0225Λon$^-$; the 36 kD recFNR was purified as described previously.[5] All other materials were obtained as noted in earlier publications,[6] or were of AR grade.

UV−vis absorbance spectra were measured with an OLIS (Bogart, GA) modified Cary-15 spectrophotometer. Fd concentrations were determined using an extinction coefficient of 9700 M^{-1} cm^{-1} at 422 nm,[13] and FNR concentrations were calculated using an extinction coefficient of 9400 M^{-1} cm^{-1} at 459 nm.[12]

Dissociation constants for complexes between the oxidized forms of Fd and FNR were measured spectrophotometrically according to a published procedure.[14] Difference spectra were recorded using dual-compartment cuvettes, and the observed spectral changes at 460 nm were fit to a hyperbolic function to obtain K_d values.

Laser flash photolysis to obtain k_{obs} values for the et reaction between Fd_{red} and FNR_{ox} was performed as described previously.[6] Briefly, a N_2 laser (PRA Model LN100, 0.1 mJ energy, 300 ps fwhm pulse duration; PRA, London, Ontario) was used to pump a dye laser (BBQ 2A368 dye, 396 nm wavelength maximum; PRA), which excited the sample containing the proteins to be studied. The photochemical reaction, in which the 5-deazariboflavin (dRf) triplet state initiates protein/protein et has been described.[15,16] In this protocol, the dRf triplet state generated by the laser flash extracts a hydrogen atom from a sacrificial donor (EDTA in this case, present in 10-fold excess) to form the dRf semiquinone radical (dRfH$^\bullet$) in less than 1 μs. This, in turn, reduces the protein (Fd_{ox}), which is present in large excess over dRfH$^\bullet$ so that pseudo-first-order conditions apply and only a single electron can enter each protein molecule. Subsequent to this reduction, protein/protein et events occur (Fd_{red} to FNR_{ox}), which are spectroscopically monitored by an optical system as described previously.[17,18] Samples used for flash photolysis contained 100 μM dRf and 1 mM EDTA in 4 mM potassium phosphate buffer, pH 7.0. Ionic strength was adjusted by addition of aliquots of 5 M NaCl. Samples without protein in 1 cm cuvettes sealed with a rubber septum were deaerated by bubbling with Ar for 1 h. When needed, Ar was blown over the sample surface to remove traces of O_2 added upon subsequent introduction of protein or NaCl to the sample. Generally 4−10 flashes were averaged. Kinetic traces were well fit by a single exponential, and were analyzed by a computer fitting procedure (Kinfit, OLIS Co., Bogart, GA). dRf was synthesized as described previously.[19]

The ionic strength dependence of protein−protein K_d values between I = 10 mM and I = 100 mM, which would be expected if the interaction were purely electrostatic, was obtained by first fitting the k_{obs} data experimentally determined in the high ionic strength region (I > 100 mM; cf. Figure 1) to the Watkins equation,[11] using only the monopole−monopole interaction term:

$$\ln k(I) = \ln k_\infty - V_{ii}X(I) \quad (3)$$

In eq 3, $k(I)$ and k_∞ are the observed rate constants at ionic strength I and at infinite ionic strength, respectively, V_{ii} is the monopole−monopole interaction parameter, and $X(I)$ is the electrostatic potential as a function of ionic strength. An interaction radius of 3.8 Å was used in the calculation of V_{ii} to optimize the fit (see ref 11 for details), which resulted in values of 49 s^{-1} for k_∞ and −10.6 for V_{ii} for the data of Figure 1. The reciprocals of the $k(I)$ values calculated from eq 3 were used to determine the shape of the ionic strength dependence in the low ionic strength region; the resulting curve was normalized to the experimental K_d value obtained at I = 12 mM in order to calculate a theoretical ionic strength dependence for K_d.

Results

The effect of ionic strength on the k_{obs} values for the reactions between Fd and natFNR and between Fd and recFNR are shown in Figure 1. It is clearly evident that both reactions show a biphasic ionic strength dependence. However, a significant difference between the two forms of FNR occurs in the low I region; the k_{obs} values for the recFNR system decrease much more dramatically, resulting in this reaction being slower by almost a factor of 4 at I = 12 mM. We have previously[9] ascribed this difference in behavior to the fact that recFNR

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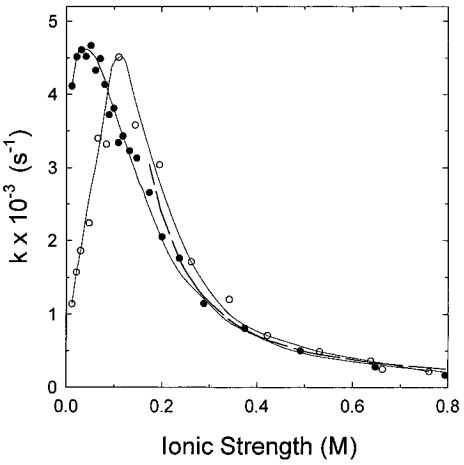

Figure 1. Dependence of pseudo-first-order rate constants on ionic strength for the reduction of native (●) and recombinant (○) FNR by Fd_{red}. For the reaction of Fd with natFNR, the reaction mixture contained 30 μM of each protein. For the reaction of Fd with recFNR, the reaction mixture contained 40 μM Fd and 30 μM FNR. Other conditions were as given in Figure 2. These data have been adapted from ref 9. The dashed line shows a fit to the k_{obs} values obtained for both proteins above $I = 180$ mM using the Watkins equation;[11] the fitting parameters were as follows: $\rho = 3.8$ Å, $V_{ii} = -10.6$, and $k_\infty = 49$ s^{-1} (see text for details).

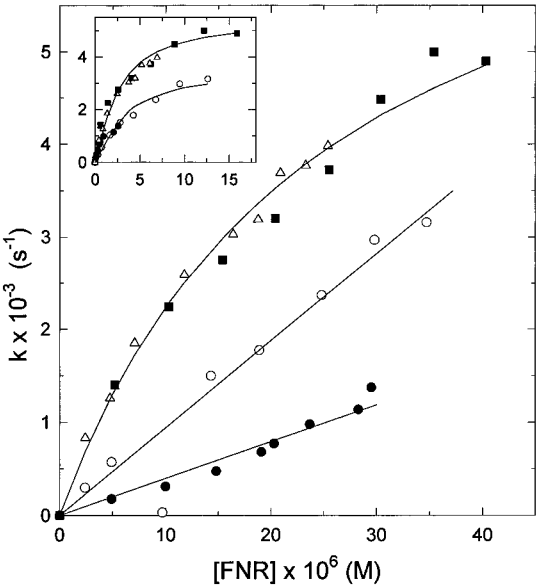

Figure 2. Pseudo-first-order rate constants for the reduction of native and recombinant FNR by wild-type Fd at ionic strengths of 12 and 100 mM. For recombinant FNR at $I = 12$ mM (●) and $I = 100$ mM (■) and for native FNR at $I = 12$ mM (○), FNR was titrated into a solution containing 30 μM Fd. For native FNR at $I = 100$ mM (△), FNR was titrated into a solution containing 40 μM Fd. All solutions were deaerated and contained 100 μM 5-deazariboflavin and 1 mM EDTA in 4 mM potassium phosphate buffer, pH 7.0. The ionic strength was adjusted with NaCl. The solid line through the Fd/recFNR and Fd/natFNR data is the theoretical fit to the data assuming a two-step mechanism (see text for details). The Fd/natFNR data are taken from ref 6. Inset: Data from main panel replotted after the FNR concentrations were corrected for preexisting $[Fd_{ox}-FNR_{ox}]$ complex, as discussed in the text.

contains six extra amino acids at the N-terminus compared to natFNR, which is due to the absence of N-terminal proteolytic cleavage of the recombinant protein in its protease-deficient *E. coli* host, a modification which does take place during the isolation procedure from the natural host *Anabaena*. The sequence of these six amino acids is TQAKAK (unpublished results). Thus, the presence of these extra residues, two of which bear a positive charge, apparently causes the highly negatively charged Fd and the net positively charged recFNR to form an intermediate complex at low I that is less optimal for et than the complex formed at high I between the two proteins, or the complex formed at low I with the natFNR. It should be noted that the N-terminal region lies just below that region of the FNR molecule which binds the FAD cofactor,[3,9] and therefore probably lies near the interaction site with Fd. The decrease in k_{obs} with increasing I which occurs for both proteins above approximately $I = 100$ mM is as expected for a collisional electrostatic interaction involving reactants of opposite charge. A nonlinear least squares fit to these portions of the data, using the Watkins equation[11] applied to the k_{obs} values for both natFNR and recFNR, is shown by the dashed curve in Figure 1. It is clear that this portion of the data is adequately fit by a model which only includes electrostatic contributions (see below for further discussion).

The dependence of k_{obs} on FNR concentration for the reduction of recFNR and natFNR by Fd_{red} at $I = 12$ and 100 mM is shown in the main panel of Figure 2. For both reactions at $I = 12$ mM, the dependence is linear, whereas at $I = 100$ mM nonlinearity is observed. Similar ionic strength dependencies have been reported previously for the reaction of natFNR with a number of recombinant Fd mutants.[6−8] The linear plots for the Fd/natFNR and Fd/recFNR systems at $I = 12$ mM yield second-order rate constants of $(1.0 \pm 0.1) \times 10^8$ M^{-1} s^{-1} and $(0.40 \pm 0.07) \times 10^8$ M^{-1} s^{-1}, respectively (Table 1: the ratio of these rate constants is consistent with the ratio of k_{obs} values for the two proteins shown in Figure 1 at $I = 12$ mM). Since saturation could not be observed under these conditions, these second-order rate constant values reflect *both* the complex formation step *and* the subsequent et step (i.e., $K_d k_{et}$; see eq 2). Despite this complication, such second-order rate constants have allowed the evaluation of the effects that site-directed mutations have on the Fd/FNR interaction.[6−8]

For the reactions of the two FNRs with Fd at $I = 100$ mM, not only is nonlinearity observed (Figure 2) but also no significant difference is found between the two proteins. The nonlinear behavior allows all of the data to be fit (solid curve through the data points) using the analytical solution[20,21] of the differential equations describing the mechanism shown in eq 2, from which K_d for the formation of the Fd_{red}/FNR_{ox} complex can be obtained, as well as a value for the limiting rate constant, k_{et}. These values are 9.3 ± 0.7 μM and 6200 ± 400 s^{-1}, respectively (Table 1), which are in satisfactory agreement with our previously published values[6] for natFNR of 6.7 μM and 5100 s^{-1}. We conclude from these data that at $I = 100$ mM the extra positive charge at the N-terminus of recFNR does not significantly influence the protein−protein interaction during the et process as it does at $I = 12$ mM, presumably due to salt ion screening of the protein charges (i.e., weakening of the electrostatic forces). As is shown also in Figure 1, at $I > 100$ mM the two proteins also behave similarly, as expected once the electrostatic charges are effectively masked.

In order to further elucidate the nature of the interaction between Fd and FNR, we have directly measured the binding constants for complex formation between the oxidized proteins over a wide ionic strength range. The binding data, the corresponding hyperbolic fits, and the calculated K_d values are shown in Figure 3. It is clear that, for the Fd/natFNR system, K_d does not change appreciably between $I = 12$ and 112 mM,

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Table 1. Kinetic Parameters for the Reduction of Native and Recombinant FNR by Fd at $I = 12$ and 100 mM[a]

		uncorrected[b]			corrected[2]	
FNR	I (mM)	$k_2 \times 10^8$ (M^{-1} s^{-1})	K_d (μM)	k_{et} (s^{-1})	K_d (μM)	k_{et} (s^{-1})
nat	12	1.0 ± 0.1	nd	nd	2.2 ± 0.3	3600 ± 400
nat	100	nd	9.3 ± 0.7	6200 ± 400	1.7 ± 0.1	5500 ± 400
rec	12	0.40 ± 0.07	nd	nd	2.2 ± 0.3	3600 ± 400
rec	100	nd	9.3 ± 0.7	6200 ± 400	1.7 ± 0.1	5500 ± 400

[a] Conditions are as given in Figure 2. [b] See text for details.

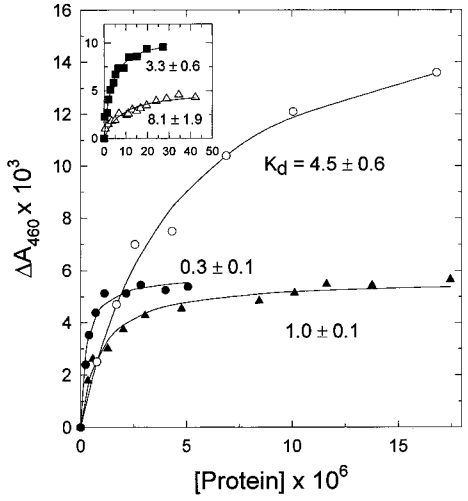

Figure 3. Spectral changes observed upon binding between native and recombinant FNR and wild-type Fd at various ionic strengths. For native FNR at $I = 12$ mM (○), FNR was titrated into a solution containing 11.3 μM Fd. For native FNR at $I = 112$ mM (△), Fd was titrated into a solution containing 10.2 μM FNR. For recombinant FNR at $I = 12$ mM (●), Fd was titrated into a solution containing 6.1 μM FNR. For recombinant FNR at $I = 50$ mM (▲), Fd was titrated into a solution containing 7.9 μM FNR. For recombinant FNR at $I = 112$ mM (■), Fd was titrated into a solution containing 10.4 μM FNR. Solutions also contained 1 mM EDTA in 4 mM potassium phosphate buffer, pH 7.0. The ionic strength was adjusted with NaCl. Solid lines through the data points are hyperbolic fits to the data assuming a 1:1 complex. Dissociation constants (μM) mfor the oxidized proteins derived from such hyperbolic fits are indicated next to each curve.

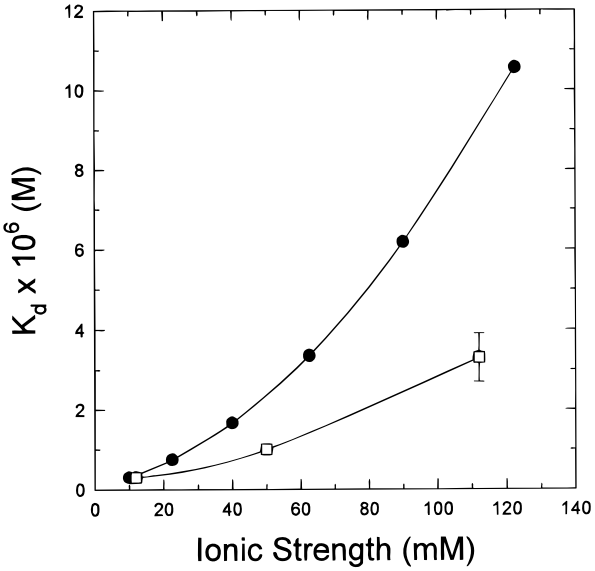

Figure 4. Theoretical (●) and experimental (○) ionic strength dependence of K_d for the Fd/recFNR protein system. The experimental values are those obtained from the data in Figure 3. Calculation of the theoretical values was performed using the parameters obtained from the Watkins equation fit to the kinetic data shown in Figure 1, as described in the text. The difference between the two curves is ascribed to the influence of hydrophobic interactions on complex stabilization.

which is consistent with K_d values for the native Fd_{red}/FNR_{ox} complex obtained from kinetic measurements.[10] For the Fd/recFNR system, K_d increases by approximately 10-fold over this same range (Figure 3). The extent of the latter increase in K_d correlates well with the large increase in the et rate constant (Figure 1) for this protein pair over this ionic strength range. Similarly, the smaller increase in the et rate constant for the Fd/natFNR system (Figure 1) corresponds with the smaller change in K_d obtained for this system. These correlations strongly suggest that the kinetic behavior and the thermodynamic behavior reflect the same underlying mechanism.

Figure 4 compares the experimentally determined values of K_d with a theoretical plot determined using the Watkins equation[11] (see Materials and Methods), which yields the ionic strength dependence of K_d which would be expected if complex stability resulted from purely electrostatic interactions. It is clear that this theoretical curve for K_d increases much more rapidly with ionic strength than the experimentally obtained values. This difference is approximately 3-fold at the highest value of I. We conclude from this that complex stability is strongly influenced by factors other than electrostatics, presumably hydrophobic interactions, which are expected to have an ionic strength dependence opposite that for ionic interactions. Thus, as I is increased, electrostatic interactions are weakened and hydrophobic interactions become stronger in the [Fd−FNR] complex.

Discussion

The above results provide strong evidence for the occurrence of both electrostatic and hydrophobic interactions between Fd and FNR during complex formation and et (Figure 4), as well as for the existence of nonproductive interactions at low ionic strengths (Figure 1). Given the magnitude of the binding constants for the $[Fd_{ox}-FNR_{ox}]$ complexes (Figure 3), a significant fraction of FNR will be present as complex at low I. It is possible to account for the decrease in k_{obs} values at $I < 100$ mM (cf. Figure 1) in a quite simple manner if one assumes that the electrostatically stabilized complex formed between the oxidized proteins (eq 4) is incapable of reacting with either $dRfH^{\bullet}$ or Fd_{red} (eqs 5 and 6), i.e., that the reactive sites of Fd and FNR are blocked in the complex and thus FNR_{red} formation can only result from direct reaction of free FNR_{ox} with Fd_{red} (eq 7).

$$Fd_{ox} + FNR_{ox} \rightleftarrows [Fd_{ox}-FNR_{ox}] \quad (4)$$

$$dRfH^{\bullet} + [Fd_{ox}-FNR_{ox}] \not\rightarrow \quad (5)$$

$$Fd_{red} + [Fd_{ox}-FNR_{ox}] \not\rightarrow \quad (6)$$

$$Fd_{red} + FNR_{ox} \rightleftarrows [Fd_{red}-FNR_{ox}] \rightarrow Fd_{ox} + FNR_{red} \quad (7)$$

This is consistent with the X-ray structure of FNR,[3] which shows a cavity near the edge of the flavin cofactor within which Fd is most probably bound. Using the measured binding constants (Figure 3), the kinetic data in Figure 2 can be corrected for the

concentration of FNR that is present as the nonproductive complex. Such corrected data are plotted in the inset to Figure 2.

For both recFNR and natFNR at $I = 100$ mM, the dependence of k_{obs} on the concentration of free FNR remains hyperbolic after correction; values of 1.7 μM and 5500 s^{-1} for K_d and k_{et}, respectively, are obtained from an analysis of these data (Table 1). Note that the value of k_{et} is essentially unchanged after correction, whereas the value of K_d is decreased from 9.3 to 1.7 μM. A smaller K_d is expected upon correction for the concentration of FNR present as nonproductive complex.

It is important to note that for both natFNR and recFNR the dependence of k_{obs} on the concentration of free FNR at $I = 12$ mM becomes hyperbolic after correction; thus, the apparent lack of saturation can simply be ascribed to competition for FNR between Fd_{ox} and Fd_{red}. Analysis of these curves yields values of 2.2 μM and 3600 s^{-1} for K_d and k_{et}, respectively (Figure 2, inset, and Table 1). Note that although the binding constants are quite similar to the corresponding values obtained for both protein pairs at high ionic strength after correction, the k_{et} values are significantly smaller. Thus, low ionic strength inhibits the et interaction between Fd_{red} and FNR_{ox}, even after correction for the concentration of preexisting complex, suggesting that the stronger electrostatic interaction forces the two proteins into a less productive orientation. When the electrostatics are weakened by the increase in ionic strength, the two types of FNR form more reactive complexes. This implies that the hydrophobic forces which begin to dominate under these conditions are more effective in producing a reactive orientation than are the electrostatic forces. It is also important to note that, at still higher ionic strengths, the weakening of the long-range electrostatic interactions apparently causes the collisions between the two proteins to become slow enough and random enough so that the frequency of productive encounters dominates the kinetics of the reaction, despite the growing importance of the shorter range hydrophobic interactions. Thus, the Watkins formalism is able to adequately fit this portion of the ionic strength dependence of k_{obs}, inasmuch as it takes into account the acceleration of the collisions by long-range Coulombic forces.

The mechanism described above is very similar to a "conformational gating" process in which there is interconversion between different conformational states each of which has different reactivities.[22] In the present system, it is the ionic strength which favors the conversion from a less productive to a more productive orientation, by changing the balance between electrostatic and hydrophobic interactions.

Previous experimental observations for the *Anabaena* Fd/FNR system are also consistent with the importance of hydrophobic interactions in the productive et complex.[6] Thus, the Fd mutant F65A was shown to react with FNR with a rate constant that was more than 10 000 times smaller than that for the et interaction involving wild-type Fd. Furthermore, this mutant was found to have a K_d more than 10 times larger than that for the wild-type protein, implying hydrophobic stabilization of the complex between the oxidized native proteins. Indeed, there is a group of hydrophobic residues located near the exposed edge of the FAD ring in FNR (J. Fontecilla, M. Frey, L. Serre, personal communication) with which F65 might interact.

Since the initial work of Salemme[23] on modeling the interaction between cytochrome b_5 and cytochrome *c*, many investigations of protein/protein et systems have concluded that electrostatic interactions are the dominant factors in properly orienting proteins within productive et complexes.[24,25] Such an interpretation is consistent with observations of a monotonic dependence of k_{obs} on *I*, an example of which is given by the oxidation of *Chlorobium thiosulfatophilum* cytochrome *c*-555 and of *Pseudomonas aeruginosa* cytochrome *c*-551 by spinach plastocyanin.[26] This type of ionic strength dependence differs dramatically from the biphasic dependence obtained for the Fd/FNR system discussed herein (Figure 1), as well as for a large number of other protein−protein systems,[26] which we have ascribed in the present study to a combination of electrostatic and hydrophobic effects. That hydrophobic interactions can stabilize protein/protein complexes has been known for many years.[27] More recently, several structural studies have indicated the involvement of hydrophobic interactions in electron transfer protein complexes. These include the crystal structure determination of the yeast cytochrome *c*/yeast cytochrome *c* peroxidase complex, which shows that hydrophobic and van der Waals interactions are the predominant forces holding this protein pair together,[28] and the crystal structure of the complex between methylamine dehydrogenase and amicyanin, which demonstrates that the interface between these two proteins is largely hydrophobic.[29] For the yeast cytochrome *c*/cytochrome *c* peroxidase system, it has also been shown by site-specific cross-linking of the two proteins that the orientation revealed by the crystal structure is et competent.[30] A number of other types of studies, including a thermodynamic study of the binding of spinach Fd to spinach FNR,[31] kinetic measurements of the cytochrome *c*/cytochrome *c* peroxidase system,[32] complex formation in the cytochrome b_5/cytochrome *c* system,[33] site-specific mutagenesis studies of the interaction between plastocyanin and photosystem I,[34−36] and measurements of the binding of NADPH-cytochrome P450 reductase to cytochrome P450,[37] have also demonstrated the importance of hydrophobic interactions in the stabilization of et protein complexes.

The present system differs from the ones described above in that an electrostatic attraction between the proteins, which is most important at low *I*, yields a *less* productive et complex. A productive orientation between the two redox proteins dominates at ionic strengths close to those found physiologically, where presumably an appropriate balance is achieved between electrostatic forces and hydrophobic interactions. In contrast to the system described herein, kinetic studies of the interaction

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between ruthenium-modified cytochrome b_5 and cytochrome c[38] demonstrated that two complexes were present, both of which were electrostatically-stabilized and et-competent. However, a recent isothermal calorimetry study of the cytochrome b_5/cytochrome c system at low ionic strength[39] showed no evidence for multiple conformers, perhaps because they are calorimetrically identical and cannot be distinguished. In this case, binding was found to be entropy-driven, presumably as a consequence of exclusion of water at the protein−protein interface. Often, as in the present study, both thermodynamic and kinetic measurements must be carried out over a wide range of ionic strengths in order to sort out the contributions of the electrostatic and hydrophobic interactions. Indeed, it may turn out that those protein/protein et systems for which *purely* electrostatic mechanisms have been invoked, and which have *not* been studied over a wide enough range of I, may need reevaluation in light of the current findings.

Acknowledgment. The work reported herein was supported in part by grants from the National Institutes of Health (DK15057 to G.T.) and from the Comision Interministerial de Ciencia y Tecnologia (BIO94-0621-CO2-01 to C.G.-M. The authors appreciate a critical reading of the paper by Dr. T. E. Meyer.

JA953662A

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